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Mechanisms of ethylenediurea (EDU) induced ozone protection: Reexamination of free radical scavenger systems in snap bean exposed to O_3

Edward H. Lee[a]*, Abha Upadhyaya[a], Madhoolika Agrawal[b], Randy A. Rowland[a]

[a]*Climate Stress Laboratory, USDA/ARS, Bldg. 046A, 10300 Baltimore Ave., Beltsville, MD 20705-2350, USA*
[b]*Centre of Advanced Study in Botany, Dept. of Botany, Banaras Hindu University, Varanasi 221005, India*

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Abstract

Ethylenediurea (EDU), N- [2-(2-oxo-1-imidazolidinyl) ethyl]-N′-phenylurea is known to prevent ozone (O_3) damage to leaf tissues. However, the mechanisms of protection are unclear. We tested the hypothesis that EDU protects against O_3 damage by scavenging hydroxyl free radicals (·OH). An in vitro study involving the use of high-performance liquid chromatography equipped with an electrochemical detector (HPLC-EC) showed that EDU does not serve as an antioxidant to remove ·OH free radicals. Effects of O_3 and EDU (soil drench) on leaf antioxidant scavenger systems (AOSS) were also studied. The first fully expanded trifoliate leaves of O_3-sensitive snap bean (*Phaseolus vulgaris* cv. Bush Blue Lake 290) was examined. Measurements were made before and after a single O_3 exposure (0.30 $\mu l\ l^{-1}$ O_3 for 3 h). Pretreatment with EDU 48 h before exposure protected against O_3-induced necrosis and chlorosis. EDU pretreatments did not alter superoxide dismutase (SOD), guaiacol-peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR) activities. However, O_3-fumigated plants (no EDU) showed elevated SOD activity with decreased GR activity. EDU-treated plants exposed to O_3 stress showed no measurable loss of GR activity. These tissues maintained high levels of total glutathione [i.e. reduced glutathione (GSH) + oxidized glutathione (GSSG)] contents, and had higher GSH/GSSG ratios than the controls at the end of 3 h exposure to O_3. These data suggest that EDU protection against O_3 damage in plants do not necessarily involve the direct stimulation or induction of antioxidative enzyme defense mechanisms. Instead, protection may result from a more general retention of chlorophyll and maintenance of GR and GSH levels during O_3 exposure. © 1997 Elsevier Science B.V.

Keywords: Antioxidative enzymes; EDU; Free radical; Glutathione; HPLC-EC; Hydroxyl radical; Ozone injury; Phytoprotection; Salicylic acid; *Phaseolus vulgaris*

1. Introduction

Ozone (O_3) is the most injurious air pollutant affecting plants in the United States [1–3]. Previously we reported that plant tolerance to O_3 can be enhanced using chemical growth regulators and antioxidant/antiozonant compounds [4–7]. Ozone sensitive snap bean plants (cv. Bush Blue Lake 290 (BBL-290)) behaved like tolerant plants after treatment with ethylenediurea (EDU), N- [2-(2-oxo-1-imidazolidinyl) ethyl]-N′-phenylurea [5, 8–11]. EDU applied systemically, through root appli-

*Corresponding author. Tel.: +1 301 5046528; fax: +1 301 5047521; e-mail: elee@asrr.arsusda.gov

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cation or as a foliar spray, converted O_3-susceptible plants into highly tolerant ones within 24 h of treatment. Up to thirty-fold increases in foliar O_3 tolerance have been reported [11]. EDU can increase the dry weight of pods after 11 weeks of ambient O_3 treatments [12]. In addition, both O_3 injury and senescence can be retarded by pretreating the plants with EDU [7, 12, 13]. Our studies showed that treatment of snap bean plants with EDU did not alter leaf lipid composition or chlorophyll and carotenoid content, but it did prevent loss (ca. 50%) of galactolipid and phospholipid caused by acute, 3 h exposure to O_3 [9]. The pretreatment does not block ethylene biosynthesis [14], nor does EDU affect photosynthetic rate or stomatal behavior [13]. However, the mechanisms of EDU protection remain unclear.

EDU-induced O_3 tolerance in snap bean plants was correlated with SOD induction [8]. Treatment with EDU has also been shown to increase SOD and catalase activities in rat lung, liver, and heart tissues without any observable toxicity [15–17]. However, Chanway and Runeckles [18] and Pitcher et al. [19] could not confirm these observations in bush bean. Ozone may affect aging processes of leaves through free radical stress [2, 3, 20]. Free radical scavengers can protect against injurious oxidants, such as superoxide anions (O_2^-), hydroxyl radicals (·OH) and hydrogen peroxide (H_2O_2) [21–24]. Free radicals have been implicated in a variety of stress injury mechanisms [21–26].

Understanding the mechanisms by which EDU induces O_3 tolerance in plants can provide valuable insights into the basis for naturally acquired O_3 tolerance in plants and factors affecting stress-induced aging in leaves. The objective of our investigation was to test in further detail the hypothesis that EDU enhances these cellular protective systems in plant tissues.

2. Materials and methods

2.1. Plant propagation

Four *Phaseolus vulgaris* L. cv. BBL-290 seeds were planted in each of seventy 15 × 15 cm (diameter × height) plastic pots containing Jiffy Mix potting mixture (Jiffy Products of America, Inc., W. Chicago, IL, USA), and pots were watered daily. After seedling emergence, plants were thinned to two per pot. They were fertilized with Peters 20-20-20 liquid fertilizer solutions applied once a week at 1.5 ml l^{-1} (Peters Fertilizer Products, W.R. Grace and Co., Fogelsville, PA, USA). Experimental plants were grown in a charcoal-filtered air greenhouse until fumigated with O_3. Environmental conditions in the greenhouse during plant growth were as follows: temperatures, 18 to 30 °C (day), 15 to 20 °C (night); relative humidity (RH), 50 to 98%; photosynthetically active radiation (PAR) of daytime maximum intensities at plant height in the greenhouse, 1500–2000 μmol m^{-2} s^{-1}.

2.2. EDU and ozone treatments

Thirty-six plants were selected from more than sixty for uniformity of height and leaf size, and divided into four treatment groups: (1) control, (2) EDU, (3) O_3, and (4) EDU + O_3. EDU treatments were made when the first trifoliate leaf was fully expanded (21 to 28 days). Eighteen pots were treated with 100 ml of 0.5 mg ml^{-1} EDU applied to the soil, while the other eighteen (control plants) received 100 ml of distilled water. Treatment was administered 48 h prior to O_3 fumigation.

EDU-treated and control plants were transferred 1 h before O_3 fumigation to a controlled environment growth chamber at 25 °C . The light intensity was 350 μmol m^{-2} s^{-1} PAR (provided by cool-white fluorescent and incandescent lamps). The RH was 60–70%. After 1 h equilibration, six non-fumigated plants were harvested from both groups (EDU-treated and untreated). The first trifoliate leaves of each plant were excised and immersed in liquid nitrogen for antioxidant analyses, and the first trifoliate leaves of the remaining plants were used for chlorophyll analysis. The remaining 12 EDU-treated and 12 untreated snap bean plants were fumigated with 599 μg m^{-3} (0.30 μl l^{-1}) O_3 for 3 h.

Ozone was generated by passing pure O_2 through a high voltage electric discharge ozonizer (O_3 generator Model 500 M, Fischer Labor- und Verfahrenstechnik, Germany). The O_3 concentration was monitored with a chemiluminescent O_3 ana-

lyzer (Bendix Corp., Ronceverte, WV, USA) which was calibrated with a Dasibi Model 1003 PC O_3 monitor (Dasibi Environmental Corp., Glendale, CA, USA). Upon completion of the O_3 treatment, first trifoliate leaf samples from six plants of both groups (i.e. O_3 and EDU + O_3) were cut, weighed, and immersed in liquid nitrogen and kept in a freezer at −80 °C until the analyses were performed.

The remaining plants (six EDU-treated and six untreated) were saved for scoring of visible injury and chlorophyll analysis 48 h after exposure. An injury rating of 0 to 10 was employed to score on the first trifoliate leaves, where 0 indicated no damage and 10 indicated 100% necrosis.

2.3. Chlorophyll extraction assays

Protective effects of EDU treatments against chlorophyll were determined from O_3-treated and untreated leaf tissues. Leaf disks were punched with a 1 cm diameter cork borer from the first fully expanded trifoliate leaves. Ten leaf disks were removed at random and used for chlorophyll determination. The disks were ground in a Polytron homogenizer with 10 ml of 80% hot ethanol and centrifuged at 500 g for 5 min. The residues were re-extracted and washed twice by centrifugation and resuspended in 80% hot ethanol. The combined supernatants were adjusted to 50 ml in volumetric flasks for chlorophyll determination using the method of Knudson et al. [27].

2.4. Antioxidative enzyme assays

Extraction of antioxidant enzymes on first trifoliate leaves of treated and untreated plants was performed using the procedures of Sankhla et al. [28]. Four treatment groups of samples (control, EDU, control + O_3, and EDU + O_3) were weighed and ground in 50 mM Tris-HCl buffer (pH 7.0), containing 1 mM EDTA, 3 mM $MgCl_2$ and 1% PVP at 4°C, using a chilled pestle and mortar. Tissue homogenates were centrifuged in a refrigerated centrifuge at 15 000 *g* for 20 min. The supernatants were used for the enzyme assays.

Catalase (EC 1.11.1.6) activity was assayed in enzyme extract reaction mixtures containing 50 mM phosphate buffer (pH 7.4). The reactions were started by adding 10 mM H_2O_2. H_2O_2 and reduction in absorbance at 240 nm was determined. Guaiacol peroxidase (GPX, EC 1.11.1.7) reaction mixture contained 50 mM phosphate buffer (pH 6.1), 1% H_2O_2, 1% guaiacol, and enzyme extract. Increase in absorbance was followed at 470 nm. Activities of both catalase and GPX were calculated using a known extinction coefficient [29].

Superoxide dismutase (SOD, EC 1.15.1.1) activity was monitored using the modified method of Dhindsa et al. [30]. Reaction mixture consisted of 50 mM phosphate buffer (pH 7.8), 13 mM L-methionine, 63 μM nitro blue tetrazolium (NBT), 2 μM riboflavin and enzyme extract. The ability of the extract to inhibit the photochemical reduction of NBT was determined at 560 nm. One unit of activity was defined as the amount of leaf extract resulting in 50% inhibition of the NBT reaction.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed according to the method of Asada [31] following the decrease in absorbance of ascorbate at 290 nm. The reaction mixture contained 50 mM potassium phosphate (pH 7.5), 0.5 mM ascorbate, 0.1 mM EDTA and 0.1 mM H_2O_2.

Glutathione reductase (GR, EC 1.6.4.2) activity was determined according to Klapheck et al. [32]. GR activity was monitored by measuring a decrease in absorbance at 334 nm resulting from the oxidation of NADPH (6.2 mM^{-1}). The assay mixture contained 0.1 M Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 mM NADPH, 1 mM oxidized glutathione (GSSG), and the leaf extract. Substrate specificity was tested by using NADH rather than NADPH.

Protein concentrations of leaf extracts were determined according to Bradford [33] using bovine serum albumin as a standard.

2.5. Analysis of glutathione in leaf tissues by HPLC

The procedure for the extraction and analysis of glutathione by HPLC was the same as previously described [34]. Glutathione was analyzed with a Shimadazu R.F. 535 HPLC (Shimadazu, Kyoto, Japan), and peaks were detected by a fluorescence detector using an excitation wavelength of 340 nm and an emission wavelength of 420 nm. Total and oxidized glutathione (GSSG) were quantified by comparing peak areas with known standards.

Reduced glutathione (GSH) was calculated by subtracting GSSG from total glutathione.

2.6. Detection of OH radicals and salicylic acid (SA) hydroxylation products by HPLC-EC

Generation, trapping, and detection of hydroxyl radical (·OH) adducted products were carried out according to the established procedures described by Floyd et al. [35] with some modification. To trap ·OH radicals 1 ml of 1 mM SA in pH 7.4 Ringer's solution was placed in each well of a six-well tissue culture cluster. To each well were added 10 mM aliquots of EDU ranging from 5 to 100 μl. A separate dish was maintained in a cold and dark area as a control. UV-induced photolysis of the reaction mixture generated the ·OH radicals. Irradiation was conducted with a UV-B lamp (Model UVM-57, Chromato-Vue; 302 nm), which was placed about 25 mm directly above the dishes. After 30 and 60 min exposures, 10 μl aliquots were removed from each well and diluted with 90 μl of 0.1 N perchloric acid. Then 10 μl of the reaction mixtures were injected directly into the HPLC system. The HPLC analyses were carried out on a Spectra-Physics P1000 System (Spectra-Physics Inc., USA) equipped with an electrochemical detector (HPLC-EC) and Bonadapak 5μ C-18 reverse-phase column (25 cm × 4.6 mm, Millipore-Waters, Milford, MA). The mobile phase for salicylate hydroxylation products consisted of 1.75 g heptanesulfonic acid and 100 mg EDTA l^{-1} in a mixture of 26% acetonitrile, 0.27% triethylamine, and 0.3% ortho-phosphoric acid. The mobile phase was prepared with deionized water and filtered through a 0.45 μm filter. The flow rate was 2.7 ml min^{-1}.

2.7. Statistical analysis

Analysis of variance (ANOVA) was performed on experimental data; statistical significance ($P < 0.05$) and was judged by the least significant difference (LSD) method. All statistical analyses were performed using software developed by Statistical Analysis System (SAS; Cary, NC).

Table 1
EDU protection of BBL-290 snap bean leaves against O_3-induced injury and chlorophyll retention, before and after O_3 treatments

Treatment[1]	Injury score[2]	Chlorophyll concentrations mg (g dry wt.)$^{-1}$ (% of control)
Control	0.0 a	8.41 (100) a
EDU	0.0 a	8.24 (98) a
Control + O_3	4.5 b	3.13 (38) b
EDU + O_3	1.0 a	7.65 (91) a

[1]Ozone concentration was 0.30 μl l^{-1} for 3 h; EDU was 0.5 mg ml^{-1} applied as a soil drench.
[2]Visual injury was scored on a scale of 0 to 10 on the first trifoliate leaves, where 0 = no injury and 10 = 100% necrosis. Data refer to the state of plants 48 h after O_3 fumigation.
Note: means separated by different letters within a column are significantly different at the 5% level ($n = 6$).

3. Results

3.1. EDU protection against O_3 damage in relation to chlorophyll and antioxidant enzymes

Pretreatment of snap bean plants with EDU dramatically reduced injury and prevented loss of chlorophyll after O_3 exposure (Table 1). Leaves of untreated controls exhibited necrosis (ca. 45% leaf injury) 48 h after fumigation, while leaves of EDU-treated plants exhibited either no visible or only minor damage (ca. 10% leaf injury). Two days after O_3 exposure, stippling, red–brown pigmentation, and fleck lesions appeared on both upper and lower surfaces of injured leaves.

Activities of GR, APX, GPX, and SOD were not significantly ($P > 0.05$) different in leaves from EDU-untreated control plants and those treated with EDU alone (Table 2). With no EDU pretreatment, O_3 fumigation significantly reduced GR activity (by 34%). However, EDU-treated plants under O_3 stress (EDU + O_3) maintained their GR activity. In both control and EDU-treated plants, SOD activity was higher after O_3 fumigation. The specific activity of catalase had slightly higher in EDU-treated plants but it declined after O_3 fumigations (Table 2).

Table 2
EDU protection of BBL-290 snap bean leaves against O_3-induced injury and antioxidant enzymes, expressed on protein basis, before and after O_3 treatments[1]

Treatment[2]	Glutathione reductase (GR) (μmol min^{-1} mg^{-1} protein)	Ascorbate peroxidase (APX) (μmol min^{-1} mg^{-1} protein)	Guaiacol peroxidase (GPX) (μmol min^{-1} mg^{-1} protein)	Catalase (μmol min^{-1} mg^{-1} protein)	Superoxide dismutase (SOD) (units mg^{-1} protein)
Control	0.18 a	0.57 a	0.25 a	28.8 ab	17.7 b
EDU	0.16 a	0.51 a	0.22 a	31.7 b	17.0 b
Control + O_3	0.11 b	0.56 a	0.25 a	29.5 ab	23.3 a
EDU + O_3	0.15 a	0.56 a	0.25 a	25.2 a	21.4 a

[1]Average of 3 duplicate assays. Data refer to the state of plants 48 h after O_3 fumigation.
[2]Ozone concentration was 0.30 μl l^{-1} for 3 h; EDU was 0.5 mg ml^{-1} applied as a soil drench.Ozone concentration was 0.30 μl l^{-1} for 3 h; EDU was 0.5 mg ml^{-1}, applied as a soil drench.
Note: means separated by different letters within a column are significantly different at the 5% level.

3.2. EDU-induced O_3 tolerance and glutathione contents

HPLC analyses of glutathione concentrations (i.e. GSH, GSSG, and total glutathione) are presented in Figure 1. The O_3 fumigated plants (control + O_3) decreased total glutathione concentrations and GSH significantly ($P<0.05$) (Fig. 1), but increased GSSG concentrations (Fig. 1), and thus decreased ratios of GSH/GSSG when compared with controls (no O_3). Pretreatment with EDU significantly ($P<0.05$) increased levels of total glutathione and GSH, and tended to decrease those of GSSG when compared with controls (Fig. 1). EDU-treated plants after O_3 stress (EDU + O_3) had significantly higher concentrations of total glutathione and GSH, but lower GSSG in comparison to control plants (no EDU + O_3) (Fig. 1). Thus,

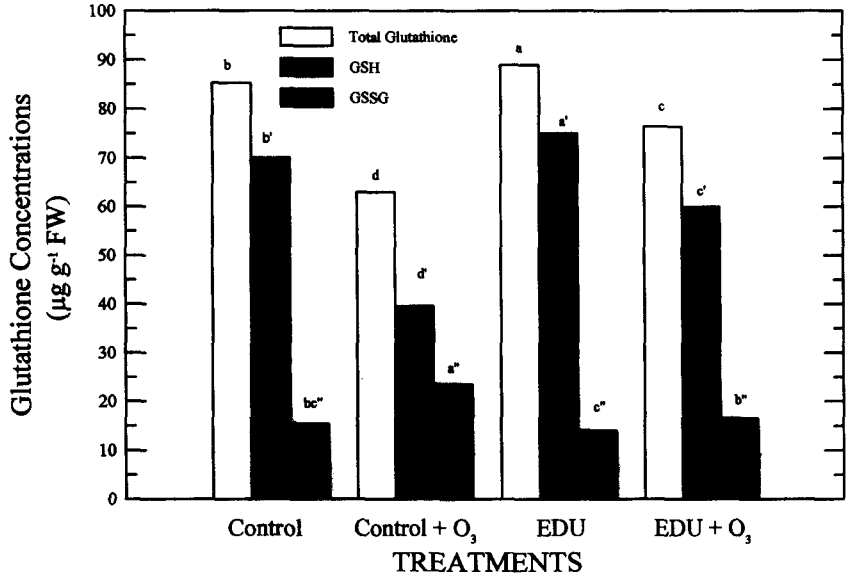

Fig. 1. Effects of EDU and ozone (O_3) on glutathione concentrations (GSH, GSSG, and total glutathione) in snap bean leaves exposed to 0 and 0.30 μl l^{-1} O_3 for 3 h at 21 days after seeds were sown. Means separated by different letters within each type of symbol are significantly different at $P<0.05$ level. Letters indicated on the top of same type of bar graphs (e.g. b = for total glutathione (open bar), b′ = for GSH (slash bar), and b″ = for GSSG (solid bar)) are significantly different at $P<0.05$ level ($n=6$).

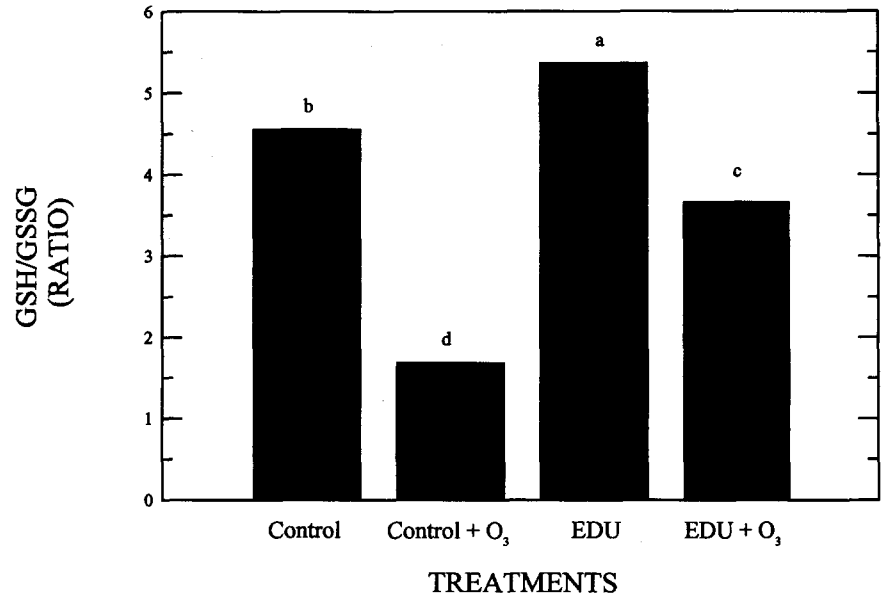

Fig. 2. Effects of EDU and O_3 on ratios of GSH/GSSG in snap bean leaves exposed to 0 and 0.30 $\mu l\ l^{-1}$ O_3 for 3 h at 21 days after seeds were sown. Means separated by different letters shown on the top of bar graphs are significantly different at $P<0.05$ level ($n=6$).

EDU-treated plants maintained the total glutathione and GSH levels during O_3 exposure. Plants pretreated with EDU had significantly higher GSH/GSSG ratios than those of the controls (no EDU, no O_3) (Fig. 2). The GSH/GSSG ratio was significantly higher in EDU+O_3 than in control+O_3 plants (Fig. 2).

3.3. Use of HPLC-EC and salicylic acid (SA) methods for trapping of OH radicals: hydroxyl radical products in the presence of EDU

By using this technique it is possible to separate the hydroxylation products of SA since it reacts with hydroxyl free radicals (·OH) to form specific hydroxylation products such as 2,3- and 2,5-dihydroxybenzoic acid (DHBA) and catechol (Fig. 3). These compounds can be detected and confirmed by the HPLC-EC procedure [35–39].

In our experiments, SA solutions with or without EDU were exposed to UV radiation for 0, 30 and 60 min to generate ·OH radicals. A typical HPLC-EC chromatogram of the SA hydroxylation products is shown in Fig. 4. It shows three peaks representing catechol (peak 1), 2,3-DHBA (peak 2), and 2,5-DHBA (peak 3). Total 2,3-DHBA formation was much higher than 2,5-DHBA.

EDU concentrations ranging from 50 to 1000 μM were ineffective in scavenging ·OH and consequently in suppressing the formation of SA hydroxylation products (Fig. 5). Increasing concentrations of EDU in SA solutions appeared to stimulate UV-

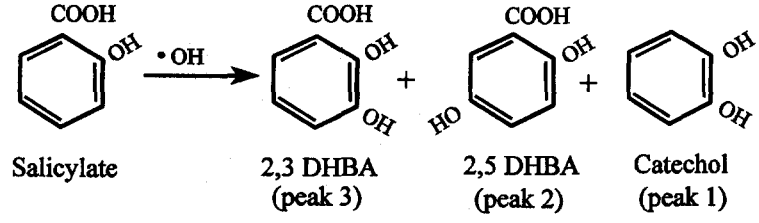

Fig. 3. Reactions of salicylate to adduct hydroxyl radicals (·OH) showing principal hydroxylation products formed, including 2,3-DHBA, 2,5-DHBA, and catechol.

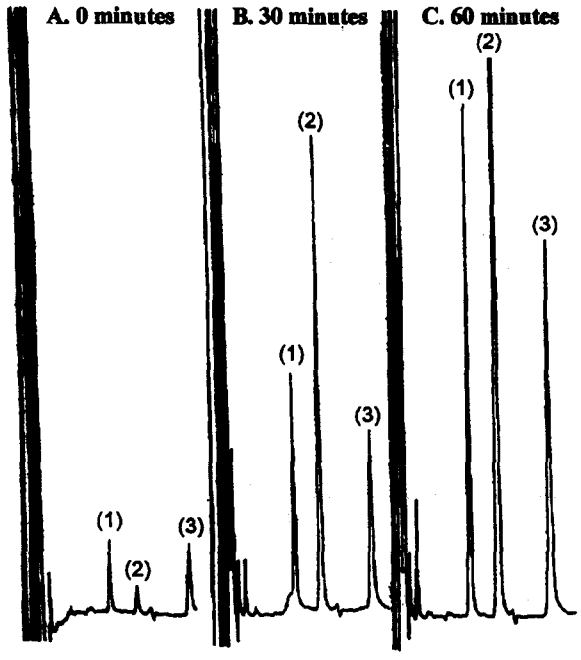

Fig. 4. Typical HPLC-EC chromatogram for in vitro trapping of hydroxyl free radicals (·OH) induced by UV (irradiation was conducted with UV-B lamp, Model UVM-57, Chromato-Vue; 302 nm), showing the effect of time on the formation of 2,3- and 2,5-DHBA in 1 μl trapping solution (1 mM sodium salicylate in Ringer's solution) after exposing 3 ml solution to UV irradiation for (a) 0, (b) 30 and (c) 60 min. Salicylic acid was used as trapping agent to capture ·OH radicals and to quantify specific hydroxylation products. Peak numbers are as follows: (1) catechol; (2) 2,3-DHBA; (3) 2,5-DHBA.

induced free radical formation. These results indicate that EDU per se does not possess free hydroxyl radical scavenging properties.

4. Discussion

The mechanisms of EDU protection against O_3 have been studied for at least 15 years [7, 8], but there is no agreement on the biochemical basis of O_3 protection [13, 18, 38, 39]. Many mechanisms have been suggested but all these have been either disputed or unconfirmed by other reports. Leaf injury and survival under O_3 stress depends on inherent protective physiological and biochemical factors [2, 3, 40]. Furthermore, there is still some doubt that O_3 can pass through the plasma membrane and have a direct effect on the cellular contents [3].

Higher activities of certain scavenger enzymes along with several antioxidants may help in protecting plants from oxidative stress including O_3 [4, 21, 38, 41, 42]. However, our studies showed no significant difference in the activities of APX and GPX between control and EDU treated plants. A similar result was also reported by Brunschon-Harti et al. [38]. Catalase activity was not significantly higher in leaves of EDU-treated control plants, but EDU-treated plants showed a significant loss of catalase activity following O_3 fumigation. Overall, EDU produced no changes in catalase activity that would account for its protective action in O_3-treated tissues.

Glutathione functions in the stabilization of antioxidative enzymes and detoxification of active oxygen species [41, 43]. EDU pretreatment that markedly reduced leaf injury and retained chlorophyll also prevented the loss of glutathione in O_3-fumigated plants. EDU-treated plants under O_3 stress (EDU + O_3) showed no significant loss of GR activities (Table 2) or total glutathione (GSH + GSSG) as compared to control plants (no EDU, no O_3) (Fig. 1). EDU-treated tissues (EDU + O_3) maintained high levels of glutathione and had higher GSH/GSSG ratios (Fig. 2) than the control + O_3. Leaves of untreated controls (no EDU) had lost more than 30% of their GSH and total glutathione by the end of a 3 h exposure to 0.3 $\mu l\,l^{-1}$ O_3. Lower ratios of GSH/GSSG in control plants after the O_3 exposure were associated with decreases in GSH. Declined was this ratio much less in EDU-treated plants compared to controls. EDU-treated plants maintained their foliar GSH and GSSG levels, and their GSH/GSSG ratio declined only slightly after O_3 fumigation. Since substantial amounts of glutathione reductase activity were maintained in EDU-treated leaf tissues after O_3 exposure, one would expect the GSH/GSSG ratio to be normally kept high under O_3 stress. Our data and those obtained from others [38] suggest that plants treated with EDU can maintain or synthesize more glutathione molecules. The outcome would then depend upon the speed of synthesis of GSH and its subsequent ability to play a

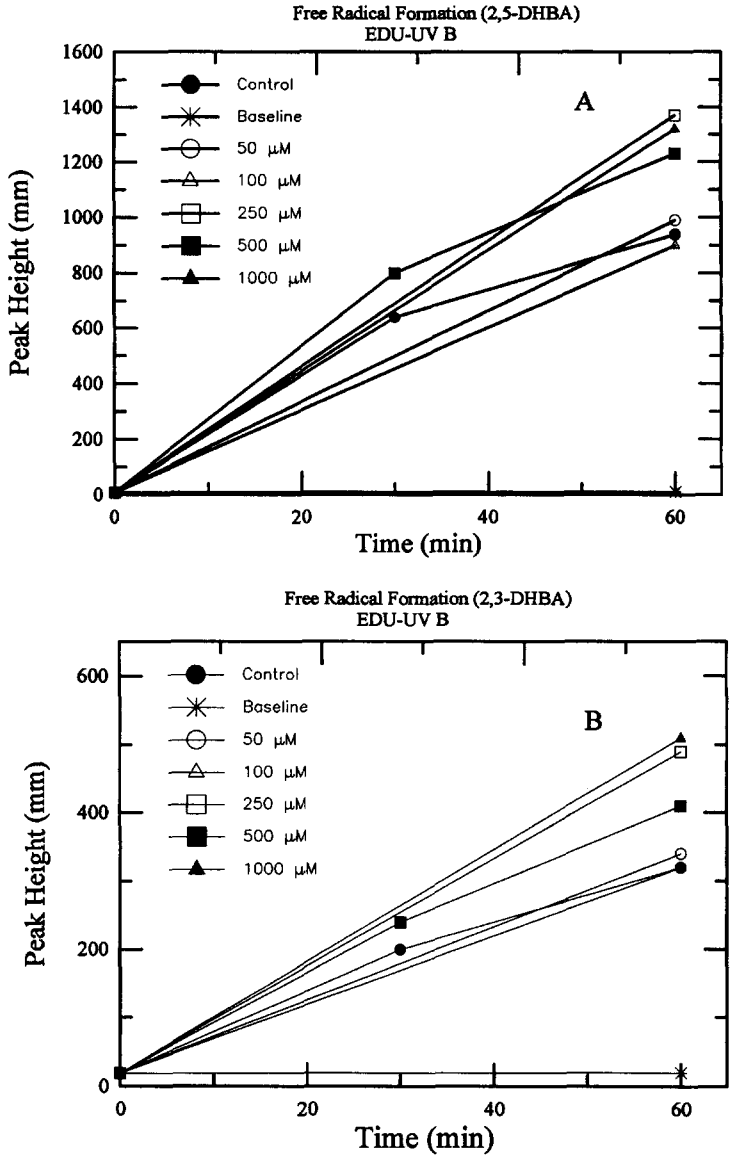

Fig. 5. Effects of EDU as an 'antioxidant' to determine the hydroxyl free radical scavenging efficiency as measured by the production of (a) 2,3-DHBA and (b) 2,5-DHBA detected by HPLC-EC in the presence of EDU. Data points represent the means of two independent experiments.

continuous role if the O_3 stress were both sustained and severe.

Since Bennett et al. [13] concluded that EDU does not induce stomatal closure in bean plants, EDU protection must have a biochemical rather than a biophysical basis, although this protection may not involve increased SOD production, as suggested by Pitcher et al. [19]. Data in Table 2 showed no differences in SOD activity between extracts from leaves of EDU-untreated controls and extracts from EDU-treated plants, either before or after the O_3 treatment. However, discrepancies in the results of previous studies [8] may be attributable to major differences in methodology. Most of

the SOD activity of green leaves is located in the chloroplasts, where it is present as a copper–zinc enzyme [23]. However, several reports of a manganese-containing dismutase in chloroplast fractions have appeared [21]. There has also been a recent report of iron-containing SOD in several higher plants [21]. Some of it is bound to the thylakoids, from which it can be released by washing in hypertonic solutions. We used partially purified SOD in the previous report [8], whereas in the present study, crude extracts were used. Both assays measured only total SOD activity and were performed without inhibitors (e.g. KCN). Results on SOD activity in this study have been confirmed by gel electrophoresis. It is not sufficiently clear to what extent EDU will affect these SOD isozymes during the partial purification process. The differences in SOD responses observed by others [19] are dependent upon the nature of the exposure to ozone and related to the developmental stages of leaves.

Considering EDU as an active antioxidant, we would expect that it should inhibit the hydroxylation of SA in solutions exposed to UV radiation [36]. Formation of hydroxylated products can also be inhibited almost completely by SOD, catalase or the iron chelator desferrioxamine [44]. The major hydroxylation products identified using HPLC-EC were 2,3-DHBA and 2,5-DHBA. A small amount of catechol was also formed by decarboxylation (Figs. 3 and 4). Quite interestingly, EDU did not suppress 2,3-DHBA and 2,5-DHBA formation (Fig. 5) and thus it probably does not suppress free radical chain initiation and/or break chain propagation reactions. The present study shows that although EDU does not act as an antioxidant to scavenge toxic hydroxyl free radicals, but it protects plants against O_3 stress. These results showed that the reductions in leaf injury and chlorophyll loss confirm earlier observations [14].

Leanderson et al. [17] reported that EDU can interfere with the generation of reactive oxygen intermediates by polymorphonuclear leukocytes. Their in vitro study showed that EDU decreased the xanthine/xanthine oxidase-induced reduction of cytochrome c by superoxide anions by directly interfering with electron transfer in the xanthine oxidase reaction. They showed that EDU can effectively diminish hydroxyl radical formation by the xanthine/xanthine oxidase reaction in the presence of chelated iron, and suggested that EDU might directly quench hydroxyl radicals. However, evidence from our present study, using SA as trapping agent of free hydroxyl radicals, did not confirm their observation.

In conclusion, our experiments showed that EDU pretreatment markedly reduced leaf injury and maintained GR and GSH levels, and retained chlorophyll in O_3-treated plants. Effective doses of EDU produced only little or no change in other antioxidative enzymes. EDU did not serve as a scavenger in the SA hydroxylation assay. Results show that EDU does not function directly as an antioxidant but helps maintain important cellular antioxidants during O_3 stress, which may be associated with conferred protection against stress. EDU has potential use for ameliorating O_3-induced oxidative tissue injury in crop plants [1, 5, 6, 12].

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References

[1] Heagle A.S. Ozone and crop yield. Annual Review of Phytopathology 1989;27:397–423.

[2] Heath R.L., Taylor G.E., Jr. Physiological processes and plant responses to ozone exposure. In: Sandermann H., Wellburn A.R., Heath R.R., editors. Forest decline and ozone. New York: Springer, 1997:317–368.

[3] Mudd J.B. Biochemical basis for the toxicity of ozone. In: Yunus M., Iqbal M., editors. Plant response to air pollution. New York: J. Wiley, 1996:276–283.

[4] Lee E.H., Jersey J.A., Gifford C., Bennett J.H. Differential ozone tolerance in soybeans and snap beans: analysis of ascorbic acid in O_3-susceptible and O_3-resistant cultivars by high performance liquid chromatography. Environmental and Experimental Botany 1984;24:331–341.

[5] Lee E.H., Rowland R.A., Mulchi C.L. Growth regulators serve as a research tool to study the mechanism of plant response to air pollution stimuli. British Society of Plant Growth Regulation 1990;20:127–137.

[6] Lee E.H., Kramer G.F., Rowland R.A., Agrawal M. Antioxidants and growth regulators counter the effects of O_3 and SO_2 in crop plants. Agriculture, Ecosystems and Environment 1992;38:99–106.

[7] Lee E.H., Bennett J.H., Heggestad H.E. Retardation of senescence in red clover leaf discs by a new antiozonant EDU, N- [2-(2-oxo-l-imidazolidinyl) ethyl]-N′-phenylurea. Plant Physiology 1981;67:347–350.

[8] Lee E.H., Bennett J.H. Superoxide dismutase: a possible protective enzyme against ozone injury in snap beans (*Phaseolus vulgaris* L.). Plant Physiology 1982;69:1444–1449.

[9] Whitaker B.D., Lee E.H., Rowland R.A. EDU and ozone protection: foliar glycerolipids and steryl lipids in snap bean exposed to O_3. Physiologia Plantarum 1990;80:286–293.

[10] Miller J.E., Pursley W.A., Heagle A.S. Effects of ethylenediurea on snap bean at a range of ozone concentrations. Journal of Environmental Quality 1994;23:1082–1089.

[11] Carnahan J.E., Jenner E.L., Wat E.K.W. Prevention of ozone injury to plants by a new protectant chemical. Phytopathology 1978;68:1225–1229.

[12] Tonneijck A.E.G., Van Dijk C.J. Effects of ambient ozone on injury of *Phaseolus vulgaris* at four rural sites in the Netherlands as assessed by using ethylenediurea (EDU). New Phytologist 1997;135:93–100.

[13] Bennett J.H., Lee E.H., Heggestad H.E. Biochemical aspects of ozone and oxyradicals: superoxide dismutase. In: Koziol M.J., Whatley F.R., editors. Gaseous air pollutants and plant metabolism. London, UK: Butterworths, 1984:413–424.

[14] Zilinskas B.A., Greenhalgh-Weidman B., Brennan E. The relationship between EDU pre-treatment and C_2H_4 evolution in ozonated pea plants. Environmental Pollution 1990;65:241–249.

[15] Stevens T.M., Boswell G.A., Jr., Adler R., Ackerman N.R., Kerr J.S. Induction of antioxidant enzyme activities by a phenylurea derivative, EDU. Toxicology and Applied Pharmacology 1988;96:33–42.

[16] Bassett D.J.P., Elbon C.L., Reichenbaugh S.S., Boswell G.A., Stevens T.M., McGowan M.C., Kerr J.S. Pretreatment with EDU decreased rat lung cellular responses to ozone. Toxicology and Applied Pharmacology 1989;100:32–40.

[17] Leanderson P., Zackrisson A.L., Tagesson C., Boswell G.A., Kerr J.S., Bassett D.J.P. EDU decreases polymorphonuclear leukocyte production of reactive oxygen intermediates. Research Communications in Chemical Pathology and Pharmacology 1994;84:133–141.

[18] Chanway C.P., Runeckles V.C. Effect of ethylene diurea (EDU) on ozone tolerance and superoxide dismutase activity in bush bean. Environmental Pollution 1984;35:49–56.

[19] Pitcher L.H., Brennan E., Zilinakas B.A. The antiozonant ethylenediurea does not act via superoxide dismutase induction in bean. Plant Physiology 1992;99:1388–1392.

[20] Kangasjarvi J., Talvinen J., Utricanainen M., Karjalainen K. Plant defense systems induced by ozone. Plant, Cell and Environment 1994;17:783–794.

[21] Bowler C., Montagu M., Van Inze D. Superoxide dismutase and stress tolerance. Annual Review Plant Physiology and Molecular Biology 1992;43:83–116.

[22] Sharma Y.K., Davis K.R. Ozone-induced expression of stress-related genes in *Arabidopsis thaliana*. Plant Physiology 1994;105:1089–1096.

[23] Foyer C.H. Oxygen metabolism and electron transport in photosynthesis. In: J.G. Scandalios, editor. Oxidative stress and the molecular biology of antioxidant defenses. Plainview, New York: Cold Spring Harbor Lab., 1997:587–621.

[24] Wellburn A.R., Wellburn F.A.M. Air pollution and free radical protection responses of plants. In: J.G. Scandalios, editor. Oxidative stress and the molecular biology of antioxidant defenses. Plainview, New York: Cold Spring Harbor Lab., 1997:861–876.

[25] Fridovich I. Biological effects of the superoxide radical. Archives of Biochemistry and Biophysics 1986;247:1–11.

[26] Larson R.A. Plant defenses against oxidative stress. Archives of Biochemistry and Physiology 1995;29:175–186.

[27] Knudson L.L., Tibbitts T.W., Edward G.E. Measurement of ozone injury by determination of chlorophyll concentration. Plant Physiology 1977;60:606–608.

[28] Sankhla N., Davis T.D., Upadhyaya A., Sankhla D., Walser R.H., Smith B.N. Growth and metabolism of soybean as affected by paclobutrazol. Plant Cell Physiology 1985;26:913–921.

[29] Maehly A.C., Chance B. The assay of catalase and peroxidase. In: Glick D., editor. Methods of biochemical analysis, vol. I. New York: Interscience, 1954:357–424.

[30] Dhindsa R.S., Plumb-Dhindsa P., Thorpe T.A. Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation and decreased levels of superoxide dismutase and catalase. Experimental Botany 1981;32:93–101.

[31] Asada K. Ascorbate peroxidase: a hydrogen peroxide-scavenging enzyme in plants. Physiologia Plantarum 1992; 85:235–241.

[32] Klapheck S., Zimmer I., Cosse H. Scavenging of hydrogen peroxide in the endosperm of *Ricinus communis* by ascorbate peroxidase. Plant Cell Physiology 1990;31:1005–1013.

[33] Bradford M.M. A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry 1976;72:248–254.

[34] Agrawal S.B., Agrawal M., Lee E.H., Kramer G.F., Pillai P. Changes in polyamine and glutathione contents of a green alga, *Chlorogonium elongatum* (*Dang*) *France* exposed to mercury. Environmental and Experimental Botany 1992;32:145–151.

[35] Floyd R.A., Watson J.J., Wong P.K. Sensitive assay of hydroxyl free radical formation utilizing high pressures liquid chromatography with electrochemical detection of phenol and salicylate hydroxylation products. Journal of Biochemical and Biophysical Methods 1984;10:221–235.

[36] Chiueh C., Krishna G., Tulsi P., Obata T., Lang K., Huang S., Murphy D. Intracranial microdialysis of salicylic acid to detect hydroxyl radical generation through dopamine autoxidation in the caudate nucleus: effect of MPP^+. Journal of Free Radicals in Biology and Medicine 1992;13:581–583.

[37] Kaur H., Halliwell B. Detection of hydroxyl radicals by aromatic hydroxylation. Methods in Enzymology 1994; 233C:67–82.

[38] Brunschon-Harti S., Fangmeier A., Jager H.-J. Effects of ethylenediurea and ozone on the antioxidative systems in beans (*Phaseolus vulgaris* L.). Environmental Pollution 1995;90:95–103.

[39] Eckardt N.A., Pell E.J. Effects of ethylenediurea (EDU) on ozone-induced acceleration of foliar senescence in potato (*Solanum tuberosum* L.). Environmental Pollution 1996;92:299–306.

[40] Lee E.H. Plant resistance mechanisms to air pollutants: circadian rhythms in ascorbic acid production in response to ozone stress. Chronobiology 1991;8:93–102.

[41] Alscher R.G., Hess J.L., editors. Antioxidants in higher plants. Boca Raton, FL: CRC, 1993.

[42] Asada K. The role of ascorbate peroxidase and monodehydroascorbate reductase in H_2O_2 scavenging in plants. In: J.G. Scandalios, editor. Oxidative stress and the molecular biology of antioxidant defenses. Plainview, New York: Cold Spring Harbor Lab., 1997:715–735.

[43] Rennenberg H. Processes involved in glutathione metabolism. Seminar Series 1995;56:155–171.

[44] Kaur H., Halliwell B. Salicylic acid and phenylalanine as probes to detect hydroxyl radicals. In: Punchard N.A., Kelly F.J., editors. Free radicals: a practical approach. New York: Oxford University, 1996:101–116.